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Immunohistochemical analysis of Ki-67, p53 and Bcl-2 expression related to histological features in gastroesophageal reflux disease

Gastroözofageal reflü hastalığında özofagus biyopsilerindeki histolojik özellikler ile Ki-67, p53 ve Bcl-2 ekspresyonunun immunohistokimyasal analizi

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Background/aims: *The endoscopic and histologic findings of gastroesophageal reflux disease are usually indistinct. The current study was designed to define accurately the histology in gastroesophageal reflux disease and to develop a hypothesis that reflux produces immunohistochemical changes.* ***Methods:*** *The study was based on the examination of endoscopic esophageal biopsy specimens obtained from 20 patients with evidence of reflux with 24-hour pH-meter monitoring and from 20 control subjects without clinical or endoscopic reflux. The pathogenesis of reflux esophagitis was discussed by comparing the histopathologic changes with determined Ki-67, p53 and Bcl-2 immunoreactivity.* ***Results:*** *In this study, the presence of esophagitis was determined endoscopically in only 55% of the patients with gastroesophageal reflux disease, while microscopic esophagitis was detected in 60% of them. No correlation was found between presence of endoscopic esophagitis and microscopic esophagitis in the patients with gastroesophageal reflux disease. There was a significant difference between control cases and the patients according to histological parameters, which included basal activity (p=0.006), height of papillae (p=0.006), intraepithelial neutrophils (p=0.000), intraepithelial eosinophils (p=0.006), congestion (p=0.001), and dilated intercellular spaces (p=0.006). Immunohistochemically, there was a significant difference in the expression of p53 and Ki-67 between the three study groups (patients with/without microscopic esophagitis, controls) (p<0.05). However, there was no difference in Bcl-2 between the patients with reflux and control cases.* ***Conclusions:*** *In this study, we considered that microscopic esophagitis does not always accompany reflux, and the lack of reliable diagnostic histologic criteria is still a serious problem for pathologists. Immunohistochemically, an increase in cell proliferative activity and p53 protein accumulation to repair oxidative DNA damage related to reflux were observed. However, the close Bcl-2 immunoreactivity in all groups that was indicated by a weak positivity suggests that the inhibition of apoptosis may not be involved in reflux esophagitis.*

Key words*:* Gastroesophageal reflux disease, Ki-67, p53, Bcl-2

Amaç: *Gastroözofageal reflü hastalığında endoskopik ve histolojik bulgular genellikle belirsizdir. Yaptığımız bu çalışma, gastroözafageal reflü hastalığı özofagus biyopsilerinde saptanan histolojik bulguları ayrıntılı olarak tanımlamak ve reflü sonucu ortaya çıkan immunohistokimyasal değişiklikleri değerlendirmek üzere planlanmıştır.* ***Yöntem:*** *Çalışmada klinik ve endoskopik değerlendirmede reflü saptanmayan 20 kontrol olgusu ile klinik-endoskopik ve 24 saatlik pH-metre ölçümleri sonucunda reflüsü olduğu saptanan 20 gastroözafageal reflü hastalığı olgusunun endoskopik özofageal biyopsi örnekleri incelemeye alınmıştır. Reflü özofajit patogenezi histopatolojik bulgular eşliğinde Ki-67, p53 ve Bcl-2 immunoreaktivitesi ile birlikte tartışılmıştır.* ***Bulgular:*** *Endoskopik özofajit gastroözafageal reflü hastalığı bulunan olguların yalnızca %55'inde gözlenirken, mikroskopik özofajit olguların %60'ında saptanmıştır. Reflüsü bulunan olgularda, endoskopik özofajit ve mikroskopik özofajit varlığı açısından anlamlı bir ilişki saptanmamıştır. Histolojik parametrelere göre kontrol olguları ve gastroözafageal reflü hastalığı olguları anlamlı derecede farklılık göstermektedir: bazal aktivite artışı (p=0.006), papilla yüksekliği (p=0.006), intraepiteliyal nötrofil infiltrasyonu (p=0.000), intraepiteliyal eozinofil infiltrasyonu (p=0.006), konjesyon (p=0.001) ve genişlemiş intersellüler mesafeler (p=0.006). İmmunohistokimyasal olarak, p53 ve Ki-67 ekspresyonu açısından kontrol grubu, reflü+histolojik özofajit grubu ve reflü+normal histoloji grubu arasında anlamlı bir fark saptanmıştır (p<0.05). Bcl-2 immunoreaktivitesi ise reflü grubu ve kontrol grubu arasında bir farklılık göstermemiştir.* ***Tartışma:*** *Çalışmamız, mikroskopik özofajitin daima reflüye eşlik etmediğini ve patologlar için güvenilir tanısal histolojik kriterlerdeki yetersizliğin halen ciddi bir problem olduğunu düşündürdü. İmmunohistokimyasal olarak, reflüye bağlı olarak gelişen oksidatif DNA hasarını onarmak üzere, özofagus epitelinde hücre proliferasyon aktivitesinde artış olduğu ve p53 proteininin hücre içinde birikime uğradığı görüldü. Tüm gruplarda Bcl-2 immunoreaktivitesinin benzer şekilde zayıf bir pozitiflik göstermesi, apoptozis inhibisyonunun reflü özofajitte yer almayabileceğini düşündürdü.*

Anahtar Kelimeler: Gastroözofageal reflü hastalığı, Ki-67, p53, Bcl-2

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic disorder that develops by the flow of gastroduodenal contents back to the esophagus. The diagnosis of GERD is based on the combination of clinical symptoms, endoscopic appearance, pH monitoring, and histologic findings (1). Most of the patients with gastrointestinal system complaints may also present the clinical findings of GERD. The most notable complaints in GERD are heartburn, regurgitation, dysphagia, odynophagia, weight loss, nausea, and vomiting. However, in addition to these complaints primarily associated with the esophagus, some other symptoms involving the oral cavity, larynx, pharynx, sinuses, and lungs may also appear (2, 3). Those who are over the age of 40 should first be evaluated by endoscopy of the upper gastrointestinal system on their admittance to the gastroenterologist. Furthermore, the endoscopic and histologic findings of patients with GERD may not be specific alone. Since GERD is a heterogeneous disease, the findings in these investigations do not always correspond. Some patients with typical symptoms have normal findings, whereas asymptomatic patients may exhibit typical endoscopic and histologic features of GERD. In fact, the "gold standard" in the definitive diagnosis of GERD is 24-hour pH monitoring (4). Although it is the most useful clinical test, this method is quite expensive and is more time-consuming. Therefore, the evaluation of the clinic, endoscopic and histologic findings in a collaborative manner is the most practical approach in the diagnosis of GERD.

In this study, patients diagnosed as GERD by 24-hour pH monitoring were also evaluated with both endoscopic and histologic findings and compared with individuals whose esophageal biopsies were normal in appearance. The pathogenesis of GERD was discussed by evaluating histologic changes and the immunoreactivities of p53, Ki-67 and Bcl-2 in all esophageal biopsies, and the contribution of immunohistochemistry to the diagnosis of GERD was researched.

MATERIALS AND METHODS

Twenty patients with GERD confirmed by clinical symptoms, endoscopic findings, histopathologic examinations, and 24-hour pH monitoring were included in this study. The control group consisted of 20 healthy subjects, who had no significant clinical reflux disease or history of heartburn, regurgitation, dysphagia, or chest pain or any other major abdominal complaints or signs of pathologic changes of the esophageal mucosa visible with conventional endoscopic examination. Patients with symptomatic GERD underwent 24-hour dual pH probe monitoring after a 12-hour starvation period to quantify the exposure of the esophagus to gastric reflux. The lower probe was positioned 5 cm above the manometrically defined upper border of the lower esophageal sphincter and the upper probe was placed 10 cm above the distal one. The patients were instructed to carry out their normal daily activities and to avoid exhausting action. Patients were asked to remain in the upright position during the daytime, and their diet was restricted to food with a pH exceeding 5. Medications that were known to affect gastrointestinal motility or acid secretion were discontinued 3 days before testing. The percentage of total monitored time during which the esophageal mucosa was exposed to a pH below 4.0 was recorded. Abnormal esophageal acid exposure was defined as a pH <4.0 for more than 4.5% of the 24-hour period (5). All patients and controls underwent upper gastrointestinal endoscopy. According to a previously published methodology, during the endoscopy, at least four biopsies were taken from 3 cm above the esophagogastric junction of each case with or without reflux (6).

Esophageal biopsies with Candida, herpesvirus or cytomegalovirus infections, any drug-related abnormality, or with systemic conditions such as collagen vascular disease or Stevens-Johnson syndrome were excluded from the study. In addition, subjects showing histologic metaplastic epithelium or the area of epithelial replacement by endoscopy were also excluded.

Specimens were immediately fixed on Hollande's fixative and oriented so that sections perpendicular to the mucosal surface could be obtained. Following ethanol dehydrating and paraffin embedding, the specimens were sectioned and then stained with hematoxylin and eosin (H&E). In addition, 5 μm thick sections were immunostained by the avidin-biotin-peroxidase complex (ABC) method. Antihuman monoclonal antibodies specific for Ki-67 (MM-1, Novocastra), p53 (DO-7, Biogenex), and Bcl-2 (124, Dako) were used in this study. After incubation with the primary antibody, the sections were reacted with 0.1% H_2O_2 in 0.1 M phosphate-buffered saline (PBS), and were treated with avidin-biotin-peroxidase complexes.

The criteria used for histological assessment of biopsy specimens were basal cell hyperplasia, papillae elongation, infiltration of neutrophils and eosinophils, dilatation of capillaries within papillae, and presence of dilated intercellular spaces (DIS) (7-9). The criteria were semiquantitatively scored as none, mild, medium, and severe on H&E-stained slides obtained from each biopsy site. A basal cell layer representing more than 15% of the full thickness of the epithelium was regarded as basal cell hyperplasia. The basal layer of the squamous epithelium was composed of smaller cells with round nuclei and basophilic cytoplasm. These cytological features of the basal layer helped in defining basal cell hyperplasia in poorly oriented samples. The elongation of papillae was estimated as papillae extending more than two-thirds of the distance to the epithelium surface. Intraepithelial neutrophils and eosinophils were evaluated in the most affected high-power field (40x). DIS were defined as an irregular round or a diffuse widening of the intercellular space and they were scored on the basis of their size and range of extension. According to the mentioned histologic features, microscopic esophagitis was recognized as the presence of a minimum of three histologic findings.

Finally, three groups were identified on the basis of symptoms, endoscopy, histology, and pH monitoring: Group I: the symptomatic reflux patients with abnormal pH who had microscopic esophagitis, Group II: the symptomatic reflux patients with abnormal pH who had no microscopic esophagitis, and Group III: the control cases with no symptoms of reflux and with a normal endoscopic and histologic appearance.

A semi-quantitative method was used to evaluate immunohistochemical reactivity of p53, Ki-67 and Bcl-2. p53 and Ki-67 reactivity was determined as nuclear staining in the expected proliferative compartment, especially over the basal zone. The density of p53 and Ki-67 was calculated as the percentage of basal layer cells. Bcl-2 is a cytoplasmic protein and showed staining in the basal and suprabasal layers, and its level was defined as the percentage of the cells in these layers.

Statistical analyses were performed using SPSS 10.0 program. Possible alterations in the immunohistochemical staining degrees of these three primary antibodies and histological parameters between the groups were analyzed by Fisher's exact probability test, while Kruskal-Wallis test was used for correlation analysis.

RESULTS

In the current study, 12 of the 20 cases with diagnosed GERD by 24-hour pH monitoring were diagnosed with microscopic esophagitis. The remaining 8 cases had either normal esophageal mucosa or nearly normal mucosa with inadequate criteria for microscopic esophagitis. Accordingly, 12 patients with microscopic esophagitis were categorized as Group I and 8 patients without microscopic esophagitis were classified as Group II. The symptom-free 20 control cases with negative endoscopic and histologic findings were assigned as Group III.

No statistical differences in age and sex were noted between the groups. Of the cases included in the study, 22 were female and 18 were male. The mean age of Group I was 41, of Group II 44, and of the Group III 54 years.

Endoscopically, in Group III (controls), the appearance of the esophageal mucosa was completely normal or nearly normal. While only 4 of 8 cases in Group II showed marked hyperemia, 7 cases of Group I had marked hyperemia and 2 of them had ulcer and erosions. Hiatal hernia was distinguished by its nontubular pouch-like appearance in 3 cases. Consequently, 11 of all cases with reflux esophagitis (55% of the patients with GERD) showed endoscopic esophagitis.

The results of histological evaluation in Group I and Group II are shown in Table 1. In Group III, except for mild congestion in two patients, no pathological findings were present. In the histopathological evaluation, microscopic esophagitis was present only in 60% of the patients who were diagnosed to have GERD through pH-meter measuring. No correlation was found between endoscopic and microscopic esophagitis in the patients with GERD. There were significant differences between the subjects with microscopic esophagitis (Group I) and without microscopic esophagitis (Group II and Group III) with respect to histological parameters such as increased basal activity ($p=0.006$), height of papillae ($p=0.006$), intraepithelial neutrophils ($p=0.000$), intraepithelial eosinophils ($p=0.006$), congestion ($p=0.001$), and DIS ($p=0.006$). No significant difference was detected in histological parameters between Group II and Group III, but a borderline insignificant result for congestion ($p=0.088$) was obtained. On the other hand, the only significant difference between Group I and Group II was seen in the basal activity increase ($p<0.05$).

Table 1. The intensity of the observed histological findings in cases with and without microscopic esophagitis

Histological findings	Intensity	Microscopic esophagitis with GERD (n=12)	No esophagitis with GERD (n=8)	p value
	None	-	6 (75%)	
Basal cell activity	Mild	7 (58%)	2 (25%)	**p=0.006**
	Medium	3 (25%)	-	
	Severe	2 (17%)	-	
	None	-	6 (75%)	**p=0.006**
Papillary height	Mild	8 (66%)	2 (25%)	
	Medium	2 (17%)	-	
	Severe	2 (17%)	-	
	None	-	8 (100%)	**p=0.000**
Intraepithelial neutrophils	Mild	7 (58%)	-	
	Medium	4 (33%)	-	
	Severe	1 (9%)	-	
	None	2 (17%)	6 (75%)	
Intraepithelial eosinophils	Mild	4 (33%)	2 (25%)	**p=0.006**
	Medium	4 (33%)	-	
	Severe	2 (17%)	-	
	None	-	3 (37.5%)	
Congestion	Mild	8 (67%)	3 (37.5%)	**p=0.001**
	Medium	4 (33%)	2 (25%)	
	Severe	-	-	
	None	-	6 (75%)	**p=0.006**
Dilated intercellular spaces	Mild	9 (74%)	2 (25%)	
	Medium	2 (17%)	-	
	Severe	1 (9%)	-	

Immunohistochemically, the expression of p53 and Ki-67 revealed a significant difference between all three groups ($p<0.05$) (Table 2) (Figures 1-4). Bcl-2 showed weak and focal staining and the percentage of its staining in our cases was 5-10%. However, the positivity of Bcl-2 staining in all three groups was closer, and therefore, no significant difference was detected between them. To define the sensitivity and the specificity of p53 and Ki-67 expression for each group, cut-off values for the diagnosis of microscopic esophagitis were determined (Table 3). Hence, it was considered that a cut-off value of 15% for Ki-67 immunoreactivity was highly specific for distinguishing the groups. Bcl-2 immunoreactivity showed low sensitivity (58.3%) and specificity (77.8%) in the diagnosis of microscopic esophagitis.

DISCUSSION

Gastroesophageal reflux disease can be diagnosed through endoscopic evaluation and pH-meter monitoring. 24-hour pH monitoring is the gold standard for the diagnosis of GERD. The sensitivity of the test is 96% and its specificity is 95%. Clinical findings of GERD correlate with lower esophageal pH <4 during more than 4.5% of a 24-hour period (4). Although chronic exposure of the lower esophagus to a pH <4 for less than 1 hour per day is commonly asymptomatic, it is likely that such exposure will damage the squamous epithelium. Pathologists have not observed any histologic change associated with subclinical or asymptomatic reflux. Furthermore, there are some asymptomatic patients who

Table 2. Positivity for the expression of p53, Ki-67, and Bcl-2 in each group

	Group I (%)	Group II (%)	Group III (%)	p value
p53	22.5	10	4.5	<0.05
Ki-67	27.5	21.25	11	<0.05
Bcl-2	5.8	2.5	2	not significant

Table 3. The cut-off values to define the sensitivity and specificity of p53 and Ki-67 expression in each group

	Cut-off value	Sensitivity	Specificity
	5.00	.727	.235
	10.00	.727	.059
p53	15.00	.636	.059
	20.00	.273	.000
	30.00	.182	.000
	40.00	.091	.000
	5.00	1.000	.824
	10.00	1.000	.529
Ki-67	15.00	1.000	.412
	20.00	.545	.176
	40.00	.091	.000

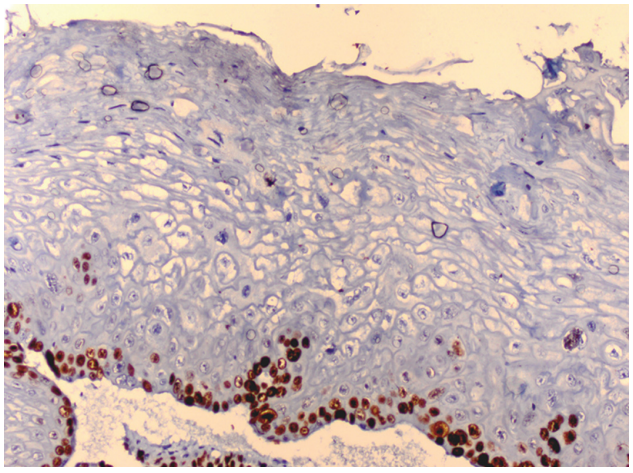

Figure 1. Immunohistochemical analysis for the expression of Ki-67 in control case (x20).

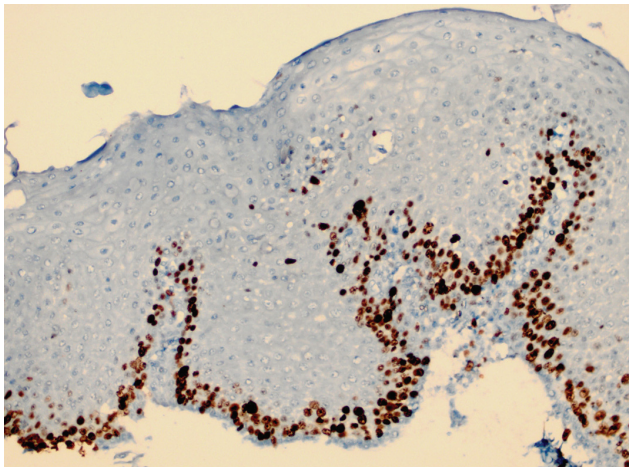

Figure 2. Immunohistochemical analysis for the expression of Ki-67 in GERD patient with microscopic esophagitis (x20).

do not have these two conditions; they have a shorter reflux period during the day or their pH value is higher than 4 (10-16). In almost half of the patients who have symptoms of reflux or whose reflux is determined pH-metrically, the esophageal mucosa is seen as normal or with only hyperemia on endoscopy. A recently published review suggests that prolonged acid exposure promotes greater damage to the esophageal mucosa, which then sequentially results in further inflammation (17). In fact, currently used histopathologic and endoscopic criteria for GERD have a low sensitivity even in patients with symptomatic reflux. The patchiness of microscopic changes is another factor, which extremely influences the diagnostic reliability of histology.

In normal conditions, the reflux of gastric or duodenal contents into the esophagus is prevented by a complex barrier. Although the etiology of reflux is multifactorial, hiatal hernia and decreased lower esophageal sphincter pressure, which constitute its most common causes, lead to the insufficiency of this barrier (18). Of the 20 cases with reflux in our study group, three had hiatal hernia but manometric evaluation could not be performed. Although our results revealed that 20 cases had GERD by pH-meter monitoring, endoscopic esophagitis was determined in only 55% of the cases. Moreover, while a great majority of them exhibited mild/moderate esophagitis, marked esophagitis characterized with erosions, hemorrhage tendency and ulcers was seen in only two cases. Consistent with the literature, these findings show that the accuracy of endoscopic appearance for the diagnosis of GERD is limited (10).

In our study, according to histopathologic parameters, microscopic esophagitis was determined in

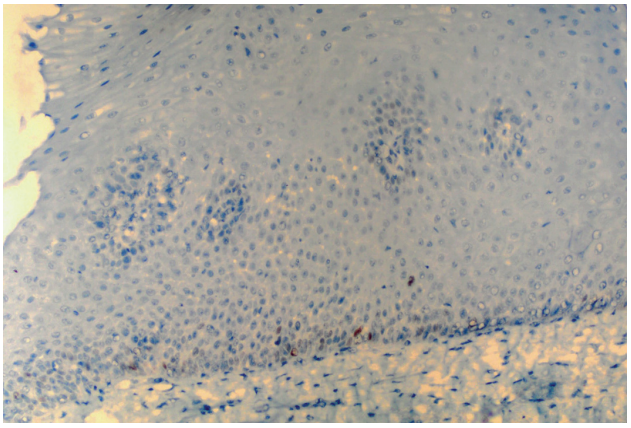

Figure 3. Immunohistochemical analysis for the expression of p53 in control case (x40).

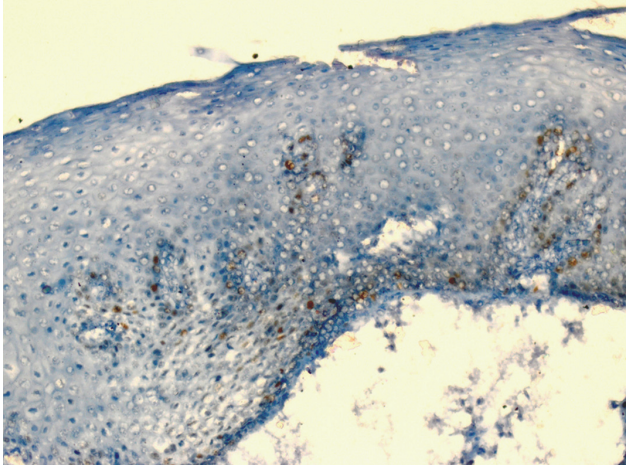

Figure 4. Immunohistochemical analysis for the expression of p53 in GERD patient with microscopic esophagitis (x40).

only 60% of the patients who were diagnosed to have GERD through pH-meter measuring. Therefore, it can be said that microscopic esophagitis does not always accompany reflux; it can emerge as a result of chronic and repeated exposure to gastric contents. However, it was seen that histological criteria are statistically significant in the evaluation of either the presence or absence of esophagitis. Although many histologic findings have been described in patients with GERD, the minimum criteria required for diagnosis remain unknown. How much abnormality suffices for the diagnosis of GERD or how one qualifies "abnormal" are matters of debate. We considered the presence of at least three histologic criteria for the diagnosis of microscopic esophagitis.

Congestion was the remarkable finding between the control cases and the cases diagnosed with reflux by pH-metry monitoring but who had histologically normal mucosa. However, in our study, it was statistically borderline insignificant. Therefore, it is maintained that congestion can be considered as the early finding of microscopic esophagitis since it is known that the dilatation and congestion observed in the capillary vessels of the papillae are insufficient for the diagnosis of reflux esophagitis (19). Finally, we have concluded that no reliable diagnostic histologic criteria have emerged totally, and the established criteria have not been detected in all patients with reflux.

As expected, it was also found in an experimental study that reactive and proliferative changes in the epithelium begin before inflammatory infiltration in reflux esophagitis (20). Since basal cell hyperplasia is the result of cell proliferation, this histological feature correlates with the immunoreactivity of Ki-67. The positivity of Ki-67 and p53 in the cases with GERD was higher than in the control group in this study. This data suggest that the proliferation step in esophageal mucosa is actively induced in association with exposure to acidic material as in the reparation responses to chronic irritation in the other tissues. It is inevitable that the activation of cell cycle-regulating proteins and increasing Ki-67 positive cells occur to repair oxidative DNA damage, which is thought to develop as a result of GERD (21-23). Therefore, the rapid turnover process in the epithelial cells probably leads to p53 mutations and p53 protein accumulation. On the other hand, a published study suggested that the expression of Bcl-2 increases in Barrett esophagus, which is thought to be the consequence of GERD, while weak Bcl-2 expression is seen only in basal cells in reflux esophagitis (24). In that study, it was concluded that the limited positivity of Bcl-2 as a marker of ongoing apoptosis in reflux esophagitis might reflect a protective mechanism counteracting increased proliferation. In Barrett esophagus, overexpression of Bcl-2 protein shows prolongation of cell survival, which may promote neoplastic progression. In these cases, p53 mutations and the activation of other oncogenes such as APC or c-erb-B2 may appear as well (25, 26). As was indicated by the weakness of Bcl-2 immunoreactivity in all our groups, the inhibition of apoptosis may not be involved in reflux esophagitis. However, it is thought that more comprehensive studies should be carried out to determine the increase in apoptosis and the definite role of apoptosis in GERD. Our data suggest that the detection of cell-cycle-related proteins such as Ki-67 or p53 might be a useful approach to discern the minimal alterations related to the reflux process.

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